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Intraperitoneal Injection of Tetracyclines Protects Mice from Lethal Endotoxemia Downregulating Inducible Nitric Oxide Synthase in Various Organs and Cytokine and Nitrate Secretion in Blood

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We have tested whether tetracyclines (TETs) are able to protect mice from lipopolysaccharide (LPS)-induced shock, a cytokine-mediated inflammatory reaction. Mice, injected with a single dose of tetracycline base (TETb; 1.5, 10, and 20 mg/kg of body weight) or doxycycline (DOXY; 1.5 mg/kg), were significantly protected from a lethal intraperitoneal injection of LPS (500 μg per mouse). TETs acted in early events triggered in response to LPS; in fact, they were no longer significantly protective if injected more than 1 h after the injection of endotoxin. LPS-treated mice protected by TETs showed a significant inhibition of tumor necrosis factor alpha (TNF-α), interleukin-1 alpha (IL-1α), and nitrate secretion in the blood, events that were directly related with the survival. In mice treated with TETs a significant decrease of inducible nitric oxide synthase (iNOS) activity was observed in spleen and peritoneal cells compared with that detected in mice treated with LPS alone. Furthermore, TETs were found to inhibit NO synthesis by peritoneal macrophages stimulated in vitro with LPS. On the contrary, TETs were unable to decrease the ability of the macrophages to synthesize IL-1α and TNF-α in vitro. These results indicate that TETs are not able to act directly on the synthesis of these cytokines, but they may modulate other pathways that could in turn be responsible for the inhibition of IL-1α and TNF-α synthesis. Altogether, these results indicate that TETs are advantageous candidates for the prophylaxis and treatment of septic shock in mice, having both antimicrobial activity and the ability to inhibit endogenous TNF-α, IL-1α, and iNOS, hence, exerting, potent anti-inflammatory effects.

Several gram-negative bacteria cause a septic shock syndrome characterized by hypotension, fever, tissue necrosis, vascular damage, and disseminated intravascular coagulation leading to multiple organ failure and ultimately death (5). The bacterial membrane component lipopolysaccharide (LPS) is responsible for this toxicity (39) inducing an overproduction of proinflammatory cytokines, such as tumor necrosis factor alpha (TNF-α) and interleukin-1 (IL-1) (13, 21, 37). The injection of appropriate doses of LPS in mice and other animals induces changes typical of the septic shock syndrome and increases the concentrations of TNF-α and IL-1 in the serum (41). The intravenous injection of TNF-α and/or IL-1 beta (IL-1β) mediates the endotoxic effect in mice (3, 37) that can be protected against endotoxin-induced shock by the administration of neutralizing antibodies against TNF-α (4) or IL-1 (8) or of receptor antagonists of these cytokines (1, 30, 35). Recently, it has been observed that the excessive generation of nitric oxide (NO) plays a relevant role in the development of septic shock (24, 40). NO is derived from the oxidation of the terminal guanidino nitrogen atom of L-arginine (27) by the NADPH-dependent enzyme NO synthase (NOS). Three isoforms of NOS have been cloned (18, 36). The Ca^{2+}-independent isoform (iNOS), which is generated by the exposition of cells to immune or inflammatory stimuli, is expressed in the widest variety of cells (7, 24, 40). The iNOS isoform is associated with tissue damage in septic shock (28), and it has been demonstrated that the in vivo administration of LPS to mice results in the production of TNF-α and IL-1, which could induce the differential expression of iNOS in the lung, heart, and spleen (8).

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On the other hand, it has been demonstrated that TNF-α and IL-1 are very important modulators of inflammatory responses. In fact, they interact with the extracellular matrix (ECM) and favor the secretion of matrix-degrading enzymes, which lead to tissue damage and the infiltration of leukocytes into inflammatory lesions (12). At the same time the interaction of these cytokines with ECM causes a modulation in the bioavailability and the activity of the cytokines themselves and may cause regulatory feedback of the initial immune reaction (2, 11, 12, 32). Thus, in monocytes, ECM binding by integrins results in the induction of tyrosine phosphorylation and subsequently in the expression of genes encoding cytokines, including IL-1 and TNF-α (11, 32). In turn, ECM glycoproteins can also induce the secretion of TNF-α (2).

Recent studies have demonstrated that antibiotics of the tetracycline family (TETs) have anti-inflammatory properties related not to their antimicrobial efficacy (15) but to their ability to inhibit the activity of protein kinase C (38), mammalian collagenases, and other related matrix metalloproteases (14, 15). Doxycycline (DOXY) and aminocycline, second-generation semisynthetic tetracycline derivatives, are more effective inhibitors of collagenase than the parent compound tetracycline (6, 15, 16). TETs were also found to inhibit bacterial endotoxins involved in periodontal disease in humans (16, 31) and to protect mice against LPS-induced lethality reducing LPS-induced TNF levels in the serum (34).

In this study, we have tested the ability of TETs to protect mice against LPS-induced shock by modulating cytokine secretion and NO synthesis.

MATERIALS AND METHODS

Mice. Ten- to 12-week-old female BALB/c mice were obtained from the Charles River Laboratory, Wilmington, Mass.

Reagents. LPS (serotype O26:B6), tetracycline base (TETb), and DOXY were obtained from Sigma (Poole, Dorset, United Kingdom). TETb was dissolved in

ethanol and diluted in phosphate-buffered saline (PBS); DOXY was dissolved in PBS. Dexamethasone (Soldesam) was from Laboratorio Farmacologico Milanese (Caronno P., Italy). Tissue culture medium consisted of RPMI-1640 (TechGen International, Les Ulis, France) supplemented with glutamine (2 mM), antibiotics (penicillin and streptomycin), and 2% fetal calf serum (FCS; Seromed, Biochrom KG, Berlin, Germany). Tissue culture plasticware was purchased from NUNC (Roskilde, Denmark). N^G-monomethyl-L-arginine hydrochloride (L-NMMA) was obtained from Bachem Feinchemikalien AG (Bubendorf, Switzerland), and L-2,3,4,5-^{3}H-arginine monohydrochloride (62 Ci/mmol) was obtained from Amersham (Milan, Italy). Other reagents were purchased from Sigma.

Induction of endotoxin shock. Mice were injected intraperitoneally (i.p.) with a 0.5-ml volume containing a dose of 500 μg of LPS (25 mg/kg of body weight). This dose was selected after accurate titration experiments on the basis of its ability to induce in mice a percentage of mortality between 70% (LD_{70}) and 90% (LD_{90}). This variability is due to the well-known difference in the sensitivities of mice to endotoxin, even when the same batch of LPS is used (5, 39). Dexamethasone, TETb, and DOXY were administered only once i.p. immediately after shock induction. Two or 4 and 12 h after LPS administration, NO and cytokine concentrations in the sera and iNOS activity in peritoneal macrophages, spleen, and lungs were determined.

NO and cytokine synthesis by LPS-stimulated PC. Peritoneal cells (PC) were collected by injecting 5.0 ml of cold medium per mouse into the peritoneal cavity under sterile conditions. Peritoneal exudate cells (PEC) were collected from mice injected i.p. 3 days previously with 3.0 ml of 2% sterilized hydrolyzed starch solution (BDH Chemicals, Poole, United Kingdom). After three washings with cold medium, the cells were resuspended at 10^6/ml and dispensed into 24-well plates (Corning Glass Works, Corning, N.Y.) for 2 h at 37°C in an atmosphere of 5% CO_2. Nonadherent cells were then removed by washing, and adherent ones were cultured with various stimuli (LPS, TETb, and DOXY). At various time intervals (24 to 48 h) the culture supernatants were collected for cytokine and NO measurement.

Assay for cytokine determination in the sera. The levels of TNF-α and IL-1α were determined by enzyme-linked immunosorbent assay (ELISA) commercial kits (Genzyme, Kocklight Ltd., Hatfield, United Kingdom) which employ the multiple antibody sandwich principle.

Assay for nitrite and nitrate concentration in plasma and supernatants. The plasma nitrate concentration was determined by reducing nitrate enzymatically using the enzyme nitrate reductase. Briefly, plasma samples were diluted 1:4 in water and 50 μl was incubated with reductase buffer (0.1 M KH_2PO_4, pH 7.5; 1 mM NADPH; 10 mM flavin adenine dinucleotide [FAD], 4 U of nitrate reductase per ml) for 3 h at 37°C. Immediately 50 μl of Griess solution (1% sulfanilamide in 5% phosphoric acid plus 1% alpha-naphytl-amine in distilled water) (9) was added for 10 min at room temperature. A standard curve of nitrate was constructed by incubating sodium nitrite (10 to 500 μM) with the reductase buffer. The absorbance was evaluated with a Titertek ELISA reader (Flow, Rockville, Md.) at 540 nm. The results are expressed as the total amount of nitrate plus nitrite (NO_2^-) per ml of plasma. In the supernatants we have evaluated the concentrations of NO_2^- using the Griess solution, and the levels of NO_2^- reflected NO synthesis.

Measurement of iNOS activity. The animals were killed, and spleen, lung and PE were collected. Afterwards, the spleen and lungs were homogenized in a glass homogenizer. The homogenates, as well as the PE, were resuspended in reaction buffer (HEPES [20 mM], EDTA [0.5 mM], dithiothreitol [1 mM]) and frozen and thawed three times. They were centrifuged at 10,000 × *g* for 30 min at 4°C, and in the supernatants the activity of iNOS, extracted from the cells, was assayed, measuring the conversion of L-^{3}H-arginine to ^{3}H-citrulline, as described by Salter et al. (33). Briefly, 20 μl of lysate was incubated with KH_2PO_4 (50 mM), valine (59.8 mM), $MgCl_2$ (2 mM), $CaCl_2$ (0.4 mM), EDTA (1.2 mM), dithiothreitol (0.8 mM), NADPH (0.2 mM), L-arginine (38 μM), L-citrulline (2 mM), TH_4Biopterin (50 mM, 50 μl), FAD (1 mM, 500 μl), and ^{3}H-arginine (25 μCi). In some tubes L-NMMA, NOS inhibitor (1 mM), was added to the other reagents. After a 15-min incubation at 37°C, the reaction was stopped by adding 0.5 ml of HEPES-Na (20 mM), pH 6, containing 2 mM EDTA. The whole reaction mixture was applied to 1-ml columns of Dowex. The radioactivity corresponding to ^{3}H-citrulline contents in 450 μl of eluate was measured by liquid scintillation counting (Beckman, Milan, Italy). The protein content of the supernatants was determined by the Coomassie blue binding method according to the manufacturer's recommendations (Pierce Chemical, Rockford, Ill.). NOS activity was expressed as pmoles of NO per milligram of protein per minute.

Statistical analysis. All of the experiments have been performed three or four times, and the results are expressed as the mean ± standard error of the mean (SEM). Some data are reported as the mean ± SEM of three or four individual experiments; others are reported as the mean ± SEM of a single representative experiment. Significance was tested by the Student *t* test by variance analysis (Student-Newmann-Keuls test). Survival curves were computed by the Kolmogorov-Smirnov and the Wilcoxon tests; otherwise the chi-square test was used. Statistical significance between survival and treated or untreated mice was calculated by the Wilcoxon test. The correlation (Pearson test) for linear regression was also used.

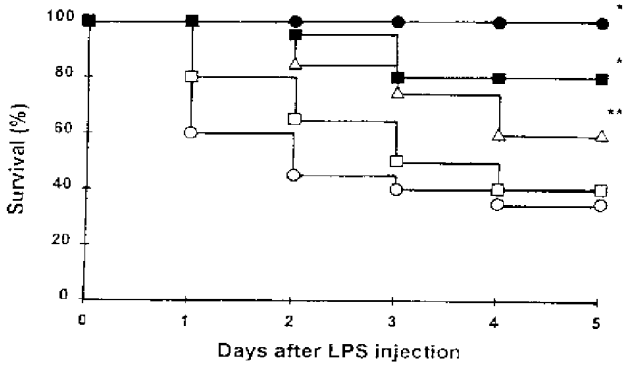

FIG. 1. TETb protects mice from lethal endotoxemia. Five groups of 20 BALB/c mice were injected i.p. with 500 μg of LPS (in 250 μl) together with 250 μl of saline (○) or an equivalent volume containing doses of TETb: 1.5 (□), 10 (■), or 20 (△) mg/kg per mouse. Death was monitored over the following 5 days. Similar results were obtained in four additional experiments. LPS plus dexamethasone was also used (30 mg/kg per mouse) (●) *, $P < 0.001$; **, $P < 0.01$; both significantly different from mice treated with LPS alone.

RESULTS

Effect of TETs on lethal endotoxemia. To determine the effectiveness of TETs on lethal endotoxemia in mice, BALB/c mice (20 per group) were injected i.p. with 500 μg of LPS in 0.25 ml of PBS, together with an additional 0.25 ml containing either PBS or different concentrations of TETb and monitored for survival rate. About 70% of animals given LPS died within 5 days. Low doses of TETb (1.5 mg/kg) had no significant effect on survival, but 10 and 20 mg of TETb per kg significantly increased protection against LPS-induced death (Fig. 1). TETb at 10 mg/kg appears to be more effective than 20 mg/kg, but the difference between the two groups does not have statistical significance. In this model LPS lethality occurs from 1 to 5 days, at which time the surviving animals are recovering and appear stable. Furthermore, we have investigated the effect of a semisynthetic derivative from TETb, DOXY, on septic shock. The most active dose of DOXY against endotoxin shock was established in titration experiments (data not shown), and it was used in a comparative test together with the more effective dose of TETb. The results clearly indicate that DOXY (1.5 mg/kg) improved the survival from endotoxin shock more than TETb (Fig. 2). We observed in kinetic studies that TETb-mediated protection was optimal when the drug was injected immediately after the LPS challenge (60 to 80%), but it was still present when the drug was administered 1 h after the LPS injection (45 to 60%). The protection decreased steadily as the TETb injection was delayed further with respect to the LPS administration, and it was similar to untreated controls by h 4 after endotoxin shock induction (10 to 20% survival). These results indicate that TETs act in the early events triggered in response to LPS.

Effect of TETs on in vivo secretion of IL-1α, TNF-α, and nitrate. We investigated if TET-mediated protection reflected a significant inhibition of TNF-α, IL-1α, and nitrate produced early by the macrophages in response to the LPS challenge. Mice injected with 500 μg of LPS and TETs contained at the time of the peak production significantly less TNF-α and IL-1α in the sera and nitrate in the plasma, respectively, than those detected in the samples from mice treated with LPS alone (Table 1). The inhibitory activity by TETs persisted even 12 h after the LPS injection. Furthermore, the survival correlates

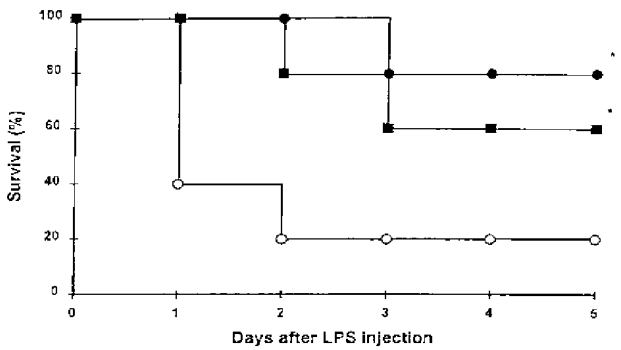

FIG. 2. DOXY protects mice from lethal endotoxemia more effectively than TETb. Twenty BALB/c mice were injected i.p. with 500 μg of LPS (in 250 μl) together with 250 μl of saline (○) or an equivalent volume containing DOXY (1.5 mg/kg per mouse) (●) or TETb (10 mg/kg per mouse) (■). Similar results were obtained in two additional experiments. *, $P < 0.001$; significantly different from mice treated with LPS alone.

with the reduction of cytokines ($r = 0.749, P < 0.05$ for TNF-α; $r = 0.739, P < 0.05$ for IL-1α) and nitrate ($r = 0.750, P < 0.05$).

Effect of TETs on iNOS induction by LPS in the lung, in the spleen, and in the peritoneal macrophages. Since the expression of the iNOS isoform may have differential induction in various organs (8), the iNOS activity was studied 2 and 12 h after LPS injection in PC, spleen, and lungs. The treatment of mice with TETb and DOXY significantly inhibited the expression of the iNOS activity in spleen and peritoneal macrophages compared to that observed in mice treated with LPS alone (Fig. 3). However, it had no effect on the iNOS activity in lungs.

Effects of TETs on the in vitro secretion of IL-1α, TNF-α, and NO. In order to explore some aspects of the mechanism involved, we tested the effects of TETs on the secretion of TNF-α and IL-1α by LPS-stimulated PC and on the synthesis of nitrate by PEC. The data shown in Table 2 indicate that TETb determined a dose-dependent inhibition of LPS-induced NO production; whereas it was ineffective on TNF-α synthesis and increased IL-1α secretion at higher doses (50 and 100 μM). Similar results were obtained with DOXY. For cytokine production we used peritoneal macrophages harvested from the peritoneal cavity, without any inflammatory stimulus in order to avoid using recently recruited and activated macrophages, but similar results were obtained when we used PEC (data not shown).

DISCUSSION

These results indicate that in mice TETs appear to be advantageous in reducing or neutralizing LPS-mediated toxicity. In view of the demonstrated roles of lymphokines (1, 13, 14) and iNOS (8, 28) in this model, the capacity of TETs to enhance survival by inhibiting some of the inflammatory products released typically by LPS-induced septic shock (TNF-α, IL-1α, and NO) constitutes an important and new means of action of the drug in addition to its antimicrobial effects.

It is at present unclear how TETs can modulate IL-1α, TNF-α, and iNOS synthesis. The results presented here show that TETs significantly reduce iNOS activity in the PC and splenocytes from BALB/c mice i.p. injected with LPS as well as inhibit NO synthesis from LPS-stimulated PC in vitro. The lack of inhibitory activity on lung iNOS suggests that NO is induced in this organ by stimuli different from those inducing NO synthesis in spleen and peritoneal macrophages that are so far unclear (8). These data explain why TETs reduce the plasma nitrate levels in vivo. Since substained high levels of NO production are expected to be damaging to the host cells and tissue (19, 22, 25) and can exert a positive feedback on the release in vivo of inflammatory cytokines (like TNF-α and IL-1α) (20), it is reasonable to believe that TETs targeting NO block the synthesis of one of the most important mediators involved in the pathogenesis of septic shock.

The contrasting activities of TETs on IL-1α and TNF-α synthesis observed in vivo and in vitro are less easy to explain. TETs are able to reduce the serum IL-1α and TNF-α levels significantly at the time of peak production (4 and 2 h after LPS challenge, respectively) and to maintain this depressive effect even 12 h after LPS injection. This indicates that they act on the synthesis rather than on the kinetics of the cytokine production. On the other hand, TETb- and DOXY-induced survival appears directly related with the reduction in the blood levels of IL-1α and TNF-α, as well as in nitrate. However, these results are in contrast with the inability of TETs to decrease TNF-α synthesis and with the ability to increase IL-1α secretion in vitro. The reason for this TET-induced IL-1α increase in contrast with TET-induced NO decrease is unclear. It is possible to hypothesize that the inhibitory activity of TETs on protein kinase C in vitro (38) may be the mechanism by which TETs increase IL-1α secretion, as reported in other experimental models (10, 26). The lack of inhibitory effects by TETs on LPS-induced TNF-α and IL-1α synthesis from peritoneal macrophages in vitro is apparently in contrast with the data of Shapira et al. (34). This difference may depend on the fact that we used mice throughout our experiments both

TABLE 1. IL-1α, TNF-α, and nitrate concentrations in the blood of BALB/c mice treated with LPS in the presence of TETb or DOXY[a]

Treatment	Concn					
	IL-1α (pg/ml) at h[b]:		TNF-α (pg/ml) at h:		$No_3^- + NO_2^-$ (μM) at h:	
	4	12	2	12	2	12
None	40 ± 10	35 ± 6	130 ± 35	110 ± 20	19 ± 6	20 ± 5
LPS	245 ± 90	100 ± 25	1,050 ± 275	250 ± 20	200 ± 50	1,250 ± 150
LPS + TETb	60 ± 13[c]	49 ± 9[c]	350 ± 150[c]	120 ± 40[c]	60 ± 10[c]	450 ± 70[c]
LPS + DOXY	50 ± 15[c]	40 ± 6[c]	200 ± 70[c]	130 ± 30[c]	39 ± 9[c]	250 ± 55[c,d]
LPS + DEX	56 ± 6[c]	39 ± 5[c]	150 ± 75[c]	100 ± 15[c]	28 ± 5[c]	150 ± 35[c,d]

[a] BALB/c mice were injected i.p. with either 500 μg of LPS or LPS concurrently with TETb (10 mg/kg) or DOXY (1.5 mg/kg) or dexamethasone (DEX) (30 mg/kg). Animals were bled at various times after the injections and monitored for survival.

[b] Point of time in which the samples for circulating cytokines and nitrate were taken.

[c] $P < 0.01$, significantly different from BALB/c mice treated with LPS alone.

[d] $P < 0.05$, significantly different from BALB/c mice treated with LPS plus TETb.

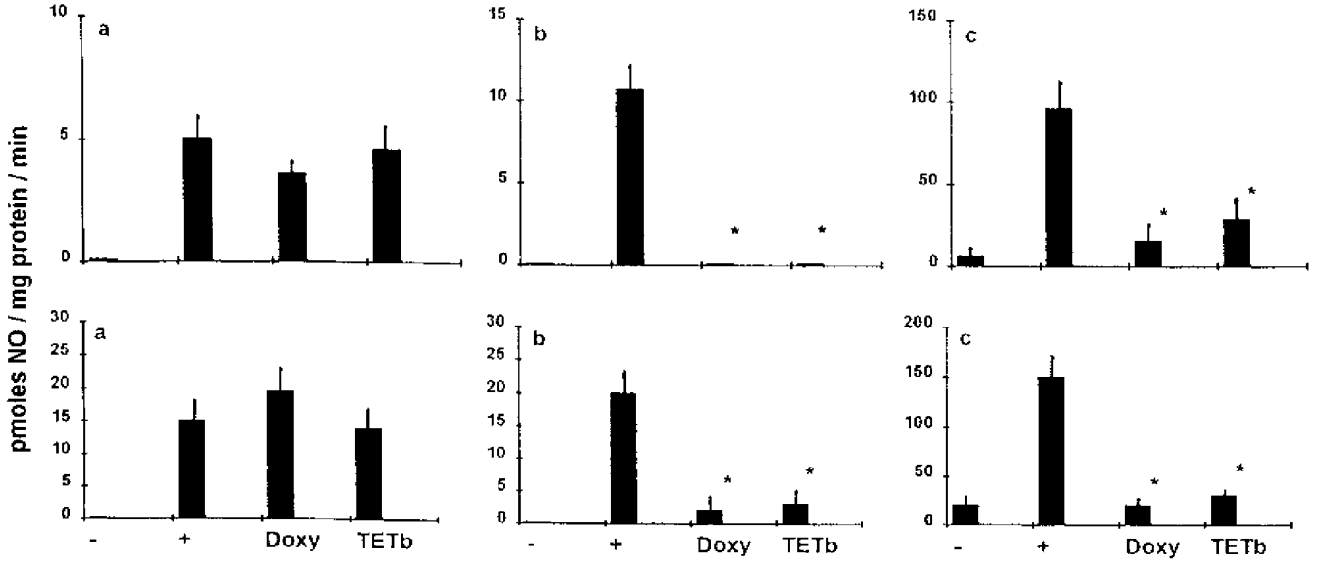

FIG. 3. Effect of LPS on iNOS level in the lung (a), spleen (b), and PC (c). Mice were injected i.p. with 500 μg of LPS and organs and cells were harvested for iNOS activity, as described in Materials and Methods, 2 (top) and 12 (bottom) h following LPS alone (+) or LPS concurrently with DOXY (1.5 mg/kg) or TETb (10 mg/kg). Control mice were injected with saline alone (−). *, $P < 0.01$; significantly different from mice treated with LPS alone. Results are the means ± SE of three experiments.

in vitro and in vivo, whereas Shapira et al. used both mice and human monocytes (34). Therefore, we consider our data more reliable than theirs, as there is a discrepancy between the rodent and human data, in particular because studies with rodents use mature macrophages, whereas studies with humans use bloodstream monocytes. On the other hand, this variance between rodent and human data indicates that the response against LPS appears peculiar in the different species and can explain why the injection of LPS in mice has been shown to be not entirely the same as infectious disease and septic shock in humans (5, 22, 24, 25, 28).

Altogether our data indicate that TETs are not capable of acting directly on the synthesis of TNF-α and IL-1α in vivo, they may modulate other pathways that could be in turn responsible for the inhibition of IL-1α and TNF-α synthesis. In line with this is the demonstration that TETs can inhibit various matrix-degrading enzymes (15, 31), reducing in such a way the secretion of TNF-α and IL-1α induced by ECM glycoprotein (2, 12) or by the binding of ECM with leukocytes (11, 12). On the other hand, it has been reported that one of the effects of LPS is to stimulate osteoblasts to secrete osteolytic factors, included IL-1α and NO, which recruit and/or activate osteoclasts producing bone resorption (17, 23). Since TETs block the activation of osteoblast procollagenase to active collagenase (29), so inhibiting the pathologic collagen breakdown, they might also reduce the secretion of IL-1α and NO, modulating this pathway. Further evidence in support of the hypothesis that the inhibition of NO by TETs may be only partially mediated through cytokines is given by the demonstration that TETs downregulate iNOS almost completely at 2 h after LPS injection, whereas the time required to induce the peak production of TNF-α and IL-1α upon LPS challenge is 2 and 4 h, respectively. The observation that DOXY determines a more significant rate of survival than TETb, even though it is not followed by different effects on the cytokines (Table 1) and nitrate production (Fig. 3), is an indication that drug-induced survival is not exclusively linked to the effect of

TABLE 2. Effect of TETb and DOXY on cytokine and NO production by PC stimulated in vitro with LPS[a]

Drug (μM)	Concn					
	TNF-α (pg/ml) at h:		IL-1α (pg/ml) at h:		NO_2^- (nmol/ml) at h:	
	24	48	24	48	24	48
None	600 ± 80	300 ± 50	130 ± 35	80 ± 15	62 ± 12	101 ± 10
TETb (25)	500 ± 30	320 ± 20	170 ± 15	110 ± 20	39 ± 9	72 ± 4
TETb (50)	590 ± 40	280 ± 20	180 ± 20[b]	140 ± 15[b]	25 ± 6	60 ± 9[b]
TETb (100)	540 ± 20	250 ± 20	189 ± 15[b]	145 ± 20[b]	20 ± 9	48 ± 10[c]
DOXY (5)	520 ± 15	290 ± 15	175 ± 10[b]	120 ± 20	35 ± 4	65 ± 6[b]
DOXY (50)	530 ± 40	280 ± 25	195 ± 15[b]	150 ± 15[b]	21 ± 6	50 ± 8[b]

[a] PC (10^6/ml) or PEC (10^6/ml) were incubated in complete RPMI medium-2% FCS with LPS (1 μg/ml) in the presence of different concentrations of drugs. After 24 and 48 h supernatants were harvested and tested for TNF-α, IL-1α, and NO levels. TNF-α and IL-1α were assayed in supernatants obtained by stimulated PC, and NO was assayed in those obtained by stimulated PEC. Data are expressed as means ± SEM of four experiments after background (medium alone) subtraction.

[b] $P < 0.05$.

[c] $P < 0.01$, significantly different from cells treated with LPS alone.

the drugs on the cytokines and nitrate but is the result of the modulation of different pathways.

In conclusion, the present results indicate that TETs are effective in the control of septic shock in mice by the modulation of the different pathways that also include the inhibition of endogenous IL-1α, TNF-α, and nitrate synthesis. The mechanism of this inhibition might be direct, as it appears in part for NO synthesis, or indirect, as is likely for IL-1α and TNF-α production. However, our data are encouraging for the use of TETs in the management of inflammatory disease, such as septic shock, even though their use in clinical human cases requires further extensive studies. DOXY, a drug with potent anti-inflammatory properties (14, 15), appears particularly useful, since it has fewer side effects than the parent compound tetracycline.

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